

The Use of the Male California Toad in the Diagnosis of Pregnancy

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SUMMARY

The California male toad, readily available in most areas of the state, and for many other reasons a better subject for test purposes than the frog, was used in 237 tests to determine the presence or absence of pregnancy in humans. Human urine was injected into the toads, and the cloacal discharge then was examined for the presence of sperm. There were only two false reactions in the series—both false negative. In subsequent tests in both these cases, the result was positive.

AMONG the forerunners of present-day frog tests for pregnancy was the technique described by Shapiro and Zwarenstein in which they utilized the female South African clawed frog (*Xenopus laevis*). Bellerby,¹ in 1934, also reported favorably on the accuracy and simplicity of this test.

Galli-Mainini,² in 1947, demonstrated that the male South American toad (*Bufo arenarum* Hensel) emitted spermatozoa after stimulation with mammalian chorionic gonadotropin. Previously, Robbins and Parker³ had demonstrated this reaction in the male frog.

That the common North American male frog (*Rana pipiens*) is an excellent and easily available test animal for the diagnosis of pregnancy has been pointedly emphasized in the work of Wiltberger and Miller⁴ (1948) and Robbins and Parker³ (1948).

The basic physiology of the Aschheim-Zondek, Friedman, rat hyperemia and frog tests consists of animal assay methods of detecting increased levels of pregnancy hormones.

In the opinion of numerous competent workers, frog tests are not only simpler, more rapid, and more economical but are apparently more sensitive in the diagnosis of early pregnancy.

In positive reactions the female frog responds by the extrusion of grossly visible ova; the male frog by the ejaculation of spermatozoa which are readily distinguishable by the microscopic examination of fluid from the external cloacal orifice.

The accuracy and speed of the male *Rana pipiens* test has been duplicated by the author of this article in a technique utilizing the male California toad (*Bufo boreas halophilus*).

The reason for the choice of this toad was its wide distribution throughout California except in deserts and highest mountains. Easier to obtain and

handle, the toad presents no problems in feeding, maintenance and (important) differentiation of sex. It is the male that does the vocalizing: A sweet falsetto trill, birdlike in character, reminding the listener of the prairie chicken, or quail. Handle the male toad, stroke it or shake it, and it will usually set up an audible protest. The female California toad is inarticulate, somewhat larger and more heavily built. During the regular breeding season (January to May) the skin of the female remains rough, whereas that of the male becomes smooth. The males usually have areas of rough, dark-colored skin on the "thumb" and inner sides of two adjacent "fingers."

Toads readily adjust themselves to artificial living conditions. For practical purposes all that is required is a box containing gravel and garden soil (light and loose for burrowing purposes) with a tray or pan filled with water at one end. The top of the box is covered with chicken wire and an electric light bulb is suspended over it in order to attract flying insects at night. This arrangement may be modified by laying the box on its side, thereby providing a wooden "ceiling" which may be hinged to facilitate handling.

Being cold-blooded vertebrates, toads, like frogs, may be induced into a state of artificial hibernation by storage in the laboratory refrigerator at temperatures not lower than 4° C. Under these conditions metabolic activity is so little that the various vital functions are carried on at the lowest possible levels and the animals are kept "fresh" for long periods. Undoubtedly, also, the ample reserve food supply of the toad (stored in fat, muscle, liver and elsewhere) is used up partly for fuel to keep the spark of life smouldering and partly for elaborating the sexual products.

The California toad, and toads in general (of the genus *Bufo*), may be distinguished from other tailless amphibians by the presence of a large raised area, the parotid gland, on each "shoulder" behind the ear membrane. The age of the adult California toad is two to three years. Other physical characteristics are: Dry skin with numerous large "warts"; upper surface grayish-green, with many large, irregular spots or streaks of black; a conspicuous streak of white extending along and down the middle of head and back; undersurface dull yellow, sometimes with numerous small black spots; hind legs only twice as long as forelegs; jaws without teeth; head-and-body length (snout to vent) ranging from 3½ to 4½ inches.

Bufo boreas halophilus is such a heavy-bodied

animal that it seldom hops in the conventional manner. When not frightened it walks in slow fashion, dragging the hind feet so that the toes are continually in contact with the ground. It breeds in ponds and quiet water of streams, irrigation ditches and "reservoirs" on farming land. Its non-breeding habitat is on or a little below the surface of the ground, wherever shelter can be found—often in gardens, parks and golf courses. It feeds on all kinds of insects and worms. It is easier to capture than the slippery, agile frog. Sufficient numbers may be obtained by advertising in local newspapers. Other good sources of supply are frog farms in the larger cities and biology classes at local schools.

Toads are carnivorous. Their favorite food consists of insects, worms, small fishes and the young of their own or allied species. Earthworms seem to be especially relished. Canned turtle food, fish food and even morsels of meat such as hamburger may be dropped directly into their buccal cavity by prying open their jaws. Toads usually retain and swallow much food whereas the frog is more liable to regurgitate it. Twice-weekly forcible feedings undoubtedly are of some value in restoring vitality to recently used animals.

Upon dissection, both male and female reproductive systems of the toad are found to be rather intimately associated with the excretory system. The male system consists of a pair of yellow oval testes, lying against the dorsal wall in contact with the kidneys. The testes have no ducts of their own, but send forth their products through the ducts of the kidneys. Attached to the anterior part of the testis is a fat-body, composed of orange-colored finger-like masses. These become very large in the summer and act as reserves of food in later months.

The female reproductive system consists of two large egg masses or ovaries. Beside each ovary is its long, large white coiled oviduct.

Toads may be killed for dissection by subcutaneous injection of chloral hydrate solution (a few crystals dissolved in 3 cc. of water) or by plunging a sharp instrument into the brain at the base of the skull.

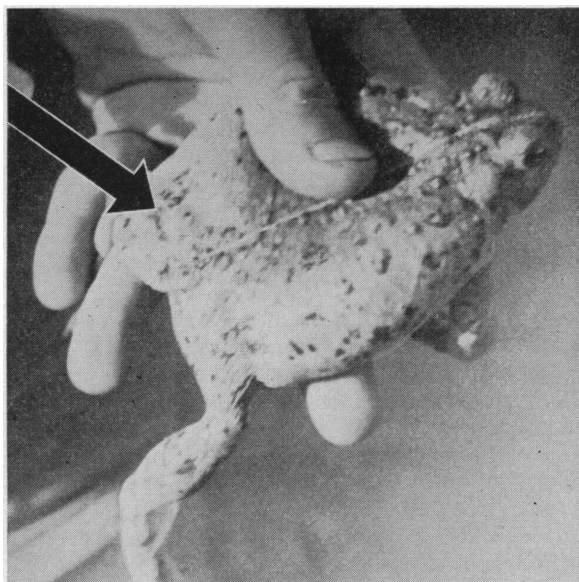
By the use of the male California toad, as with the *Rana pipiens*, laboratory diagnosis of pregnancy can be carried out within two to three hours, often sooner. The endpoint is unequivocal; sperm is either present or absent.

TECHNIQUE

The following technique is based on analyses on 237 specimens of urine from the sources shown in Table 1. Accuracy of 99 per cent was attained.

Technique.

Collect the first morning specimen of urine, which is a concentrated one, in a clean container, not necessarily sterile. Random specimens with a not too dilute specific gravity have proven satisfactory. Centrifuge, filter or let stand to clarify. Without adjustment of pH or control of temperature, inject



The male California toad (*Bufo boreas halophilus*). Recommended method of injecting urine into subcutaneous dorsal lymph sac. Needle (arrow) is directed toward median line just above the upper cloacal fold.

5 to 10 cc. subcutaneously into the dorsal lymph sac of each of a pair of male toads. Thrust the needle (22 gauge) superficially into the upper thigh muscle area, directing the point to the midline just above the upper cloacal fold near the acetabulum.

Beginning one hour later obtain fluid, using a glass pipette, from the external cloacal orifice and examine under the low power lens of the microscope with reduced light. Spermatozoa, usually actively motile, are very readily seen if present. This is a positive reaction. The long, undulating flagellum of the individual sperm is easily distinguishable when the high power lens is used.

A negative reaction is reported when no sperm is seen during a three-hour period of observation following inoculation with the test urine. In certain cases in which the reaction is negative, urine from a woman known to be pregnant is administered in order to demonstrate the ability of the toads used to respond to the presence of the chorionic hormone.

Occasionally the urine proves to be toxic, either killing the toad or rendering it very ill. If another specimen cannot be conveniently obtained, the split-dosage technique of Robbins and Parker may be employed: Two half-doses given an hour apart.

TABLE 1.—Results of Toad Tests for Frequency

Source of urine	Correct Positive	Correct Negative	False Positive	False Negative
Pregnant women	136	2
Women with amenorrhea without pregnancy	63	0
Normal women with menses	36	0
	136	99	0	2

Before giving the second injection it is advisable to make an examination of cloacal fluid, as a positive reaction may already be discernible at this stage.

NOTES

After a resting and feeding period of at least three weeks in a separate terrarium, animals which have been shown to react positively may be reused.

The toxicity of certain urines is most often due to drugs (ergot, quinine, morphine, codeine, sulfa drugs and even acetylsalicylic acid). The patient should abstain from taking drugs prior to collection of the specimen.

Blood serum and urine may be used interchangeably. It is claimed that, in the interests of a less toxic, more constant source of hormone, blood serum appears to be preferable. However, many laboratory technicians favor the use of urine over serum.

If a negative report is obtained on a specimen voided less than seven days after the first missed menstrual period, the test should be repeated later.

As the initial step in the Bufo test the author deems it wise to examine a cloacal smear prior to inoculation to rule out the spontaneous presence of sperm.

CONCENTRATION METHOD

A rapid concentration method may be used upon encountering certain urines suspected to contain a low hormone titer: Adjust entire collection of urine to a pH less than 4.5 by adding hydrochloric acid drop by drop until nitrazene paper turns sharply lemon yellow and blue litmus turns sharply red. Place all of specimen in a 1000-cc. graduated cylinder and mix with 2 volumes of acetone. Let stand or centrifuge to collect sediment. Pool sediment in centrifuge tube and wash with ether, mashing and spreading sediment along sides of tube with an applicator stick. Decant ether (or centrifuge) and run current of air over sediment until dry. Dissolve sediment with 3 to 4 cc. of water, using same applicator

stick (which has remained in tube) to disperse and break up clumps. Centrifuge and adjust supernatant to pH 5.5 or pH 6.00 with sulfosalicylic acid. Inject half-portions of this into each of a pair of male toads.

DISCUSSION

Duplicate tests utilizing *Rana pipiens* were conducted in every instance in this series of 237 tests with the California toad. Occasionally rabbits were employed to corroborate certain negative findings. No discrepancies were noted.

In this series two false negative reactions were obtained. Tests with urine specimens collected one week later, in both cases, gave positive results. In connection with the false negative reactions, various possibilities may be mentioned: (1) Low hormone titer (specimens collected too early?); (2) use of immature toads (only those definitely measuring over $3\frac{1}{2}$ inches from snout to vent should be selected); (3) presence of inhibitors or depressants due to drugs, medicines, etc.; (4) urine of very low specific gravity. The following guide for determining the dose from the specific gravity is recommended:

Specific Gravity	Dose
Under 1.012	10 cc.
1.013 to 1.019	8 cc.
1.020 to 1.029	6 cc.
1.030 and over	5 cc.

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